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Antibacterial activity evaluation of cambuí extract against multi-resistant Enterococcus faecium

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Abstract
Bacterial resistance is a global public health issue. Among these pathogens, Enterococcus faecium nosocomial has been highlighted due to its potential to cause bacteremia infections. The incidence of resistant Enterococcus faecium bacteremia has increased over time, causing high mortality rates. Hence, it led to the interest in natural drugs. Plant extracts from the Myrtaceae family have a broad-spectrum antimicrobial action. Among the plants of this family, we can highlight cambuí due to its wide variety of bioactive compounds, such as phenolic compounds. Polyphenols are bioactive molecules that can inhibit the growth of resistant bacteria such as Enterococcus faecium. For this, the present study aimed to obtain bioactive extracts from cambuí using pressurized hot water to grow inhibition of multiresistant Enterococcus faecium nosocomial. In the present study, antibacterial compounds were obtained after extraction kinetics over 120 minutes at a temperature of 50 ºC, a flow rate of 1 mL/min, and a constant pressure of 200 bar using water as the extraction solvent—spectrophotometric methods quantified flavonoids and other phenolic compounds from cambuí extracts. The TSA using the well-diffusion method was used to verify the sensitivity of the bacterium Enterococcus faecium against cambuí aqueous extract. The results showed the best extraction time was 60 minutes using 60 mL of water. The cambuí aqueous extract showed relevant flavonoids and other phenolic compounds. Hence, this extract was able to inhibit the growth of the bacterium Enterococcus faecium resistant to Ampicillin, Imipenem, Vancomycin, and Teicoplanin.

Keywords
Bacterial resistance — Enterococcus faecium — Myrciaria tenella O. Berg — Phenolic compounds — Pressurized liquid extraction

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1. Introduction

Bacterial resistance has been a significant cause of morbidity and mortality for millions worldwide. Among the most relevant nosocomial pathogens worldwide, we can highlight *Enterococcus faecium*, which causes fatal infections in elderly and immunocompromised patients [Mališová et al., 2021, da Silva et al., 2016].

This relevant public health problem is associated with the low efficiency of commercial antibacterial agents against bacterial strains, which has led to the search for new efficient antibacterial agents [da Silva et al., 2016]. For this, medicinal plant extracts have been an essential source of bioactive molecules against the growth of multiresistant bacteria [Ríos and Recio, 2005].

Extracts from plants of the *Myrtaceae* family have pharmacological properties to inhibit the growth of gram-positive and gram-negative bacteria. This broad spectrum of antimicrobial action of *Myrtaceae* plants raises interest in their use to obtain antibacterial molecules [de Paulo Farias et al., 2020, Borges et al., 2014].

Among the exotic plants of the Brazilian northeast of the *Myrciaria* genus, we can highlight the cambuizeiro (*Myrcia-*

ria tenella O. Berg), which produces a fruit (cambuí) with high added value, which has been consumed in nature, in the form of drinks and jellies [Seraglio et al., 2018]. In addition, cambuí has bioactive compounds such as vitamins and phenolic compounds [Perioto et al., 2022, Cruz Silva et al., 2020].

Flavonoids and other phenolic compounds have potential antimicrobial effects [Takshak and Agrawal, 2019]. With this, the pharmaceutical industries have awakened the need to search for more efficient methods for extracting bioactive compounds [Azmir et al., 2013, Zhang et al., 2020]. It is observed that the quality of natural products can present significant differences in their chemical composition, according to the solvent and the extraction process used [Santos et al., 2018].

Pressurized liquid extraction (PLE) is a potential method to obtain natural compounds, as it allows the use of solvents under high pressure, which favors the penetration of the solvent into the plant matrix in a way that would not be possible under atmospheric pressure. Thus, a more excellent removal of bioactive compounds from the plant material studied [Trentini et al., 2017]. In addition, this technology allows kinetic studies of extraction yield that will provide the amount of solvent and extraction time necessary to obtain natural compounds better [Hall et al., 2018, Sovová, 2005].

The extraction yield by different solvents such as water, chloroform, hexane, ethanol, and ethyl acetate demonstrates a distinct ability to obtain bioactive compounds [Patel et al., 2016]. Among these solvents, water has been highlighted for its efficiency in separating phenolic compounds from plant matrices and for being non-toxic, naturally abundant, and inexpensive [Plaza and Turner, 2015, Flórez et al., 2015].

The present study aimed to obtain antibacterial cambui fruit extract by pressurized water extraction and evaluated its potential to grow inhibition of mult-resistant Enterococcus faecium nosocomial.

2. Experimental

2.1 Development of equipment for extracting antibacterial compounds from Cambuí

The extractions were carried out in a stainless steel extractor cell with an internal volume of 20 mL, connected to a positive displacement pump (Series III, Lab Aliança, USA), for pressurization and flow control of the extractor solvent (water). The extractor cell was inserted into an ultrasound-type bath (Ultronique, Q5.9/40A, Brazil), which provided the extraction temperature.

The pressure during extraction was monitored using a pressure transducer (5436 Würenlos, Control Huba, Switzerland) connected to a pressure indicator (N1500, Novus, Brazil). Finally, the solvent/extract mixture was collected from a needle-type valve connected to the extractor cell output. The best details of the equipment can be seen in Figure 1.

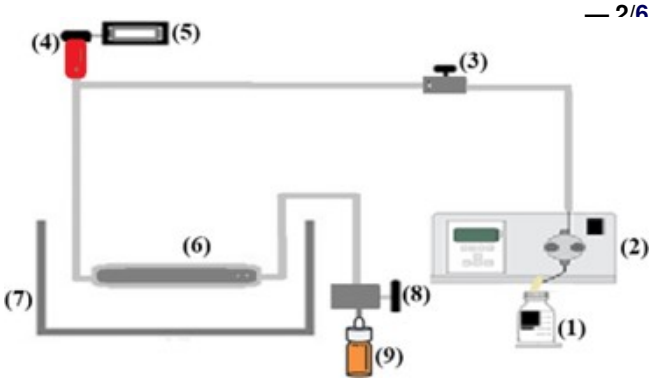

Figure 1. Schematic diagram of the extraction equipment developed to obtain antibacterial biomolecules. Where: 1 - Solvent reservoir; 2 - Positive displacement pump; 3 - Ball valve; 4 - Pressure transmitter; 5 - Pressure indicator; 6 - Extractor cell; 7 - Ultrasonic bath; 8 - Needle valve; and 9 - Extract collector. The equipment is complete with 316 (1/8") stainless steel piping and electrical cabling.

2.2 Collect and Vegetal Sample Preparation

Cambuí were collected without differentiation of color and age from the native population of the Private Reserve of Natural Heritage (RPPN) of Caju, belonging to the Experimental Field of Embrapa Tabuleiros Costeiros, in the municipality of Itaporanga d'Ajuda, SE (lat - 11.116585º, log - 37.186742º).

The obtained fruits were dried in an oven with hot air circulation at a controlled temperature of 45 ºC until they reached a moisture content of about 5%. After that, they were crushed in a knife mill and separated into 16 - 32 mesh granulometry. The samples were stored in a refrigerator (-4°C) and protected from light until extraction.

2.3 Process for obtaining antibacterial compounds by pressurized liquid

The extraction process with pressurized fluid was performed using 5 g of the dry plant sample, which was inserted into the extractor cell. The separation of antibacterial compounds from cambuí was performed using distilled water. The extraction time was determined based on the results obtained from the extraction kinetics in the time interval of 1, 3, 5, 10, 15, 30, 45, 60, 90, and 120 minutes.

All extractions were performed in dynamic mode, with a temperature of 50 °C, a pressure of 200 bar, and a constant flow of 1 mL/min. The extracts obtained were dried in an oven with hot air circulation at a temperature of 45 ºC until a stable weight was obtained. After drying, the extracts were stored under refrigeration at 5 ºC until the time of chemical analysis.

2.4 Determination of total phenolic compounds

The phenolic compounds of cambuí extracts were estimated using the Folin-Ciocalteu method. Briefly, in a test tube was 0.5 mL of extract from a stock solution of 100 ppm diluted in methanol) with 0.5 mL of distilled water, 2.25 mL of Folin-

Ciocalteu (7% m/v), and 1.75 mL of sodium carbonate (7.5% m/v).

Subsequently, the solution was incubated at 45 ºC for 20 min and kept at room temperature for 10 min for reading at 765 nm in a spectrophotometer (Synergy HT, BioTek, USA). Gallic acid was used as a standard for the calibration curve (5 - 140 g/mL), and the total contents of phenolic compounds were reported as mg of gallic acid equivalents per gram (mg GAE/g) of cambuí dry extract.

2.5 Determination of total flavonoids

Total flavonoids were quantified using the colorimetric method with a 10% aluminum nitrate solution (w/v). Briefly, 0.5 mL of extracts (from a stock solution of 100 ppm diluted in methanol) were used, which were mixed in a solution of 0.1 mL of aluminum nitrate at 10% (w/v) and 0.1 mL of 1 M potassium acetate.

The final volume was completed with 4.3 mL of methanol. Then, the samples were homogenized at room temperature, and the absorbance readings were performed in a spectrophotometer (Synergy HT, BioTek, USA) using a wavelength of 425 nm. A calibration curve was prepared with standard rutin solution (5-140 g/mL), and the values obtained were expressed as mg of rutin equivalents per gram (mg.RE/g) of cambuí dry extract.

2.6 Isolation of bacteria

Microorganisms were isolated from hospital blood culture discards using swabs to seed the bacteria on chocolate agar culture media. Then, the inoculum was incubated in a bacteriological oven for 24 hours at 35 °C. The isolated microorganisms were submitted to Gram staining and identified as Gram-positive cocci.

Subsequently, the identification and susceptibility test of the isolated bacterium was performed using Beckman Coulter Micro Scan-AutoScan 4 equipment. The commercial antimicrobial agents tested to verify the sensitivity and resistance profile were Ampicillin, Gentamicin, Streptogranamines, Imipenem, Vancomycin, Teicoplanin, Linezolid, Deptomycin, Tetracycline and Sinercid.

2.7 Antimicrobial Sensitivity Test (AST)

The AST was performed using the healthy method described by Silva Santos et al. 2016 [Silva Santos et al., 2016]. Petri dishes containing Mueller-Hinton culture medium inoculated with cultures of *Enterococcus faecium* (1.0 x 108 CFU/mL) were used for this.

The plates were allowed to dry for about 5 minutes. Then 500 mg of cambuí aqueous extract (EAC) diluted in 500 µL of saline solution or only saline solution without the agent that inhibited bacterial growth (vehicle) were placed in the wells. Plates were incubated at (37 ± 2 °C) for 24 hours. After this period, the presence or absence of inhibition halos was verified.

2.8 Statistical Analysis

All results were presented with the mean and their respective standard deviation. Significant differences were assessed by ANOVA followed by Tukey's test (p 0.05) using GraphPad Prism version 5.0 statistical software, San Diego, CA.

3. Results

3.1 Obtaining a bioactive extract via pressurized fluids and determining the total polyphenol content

In Figure 2, we observe the Global Extraction Curve (GEC) of the cambuí molecules, which shows the three distinct periods of the mass transfer mechanism: Constant Extraction Rate (CER), Falling Extraction Rate (FER) and Rate of Low Extraction (RLE).

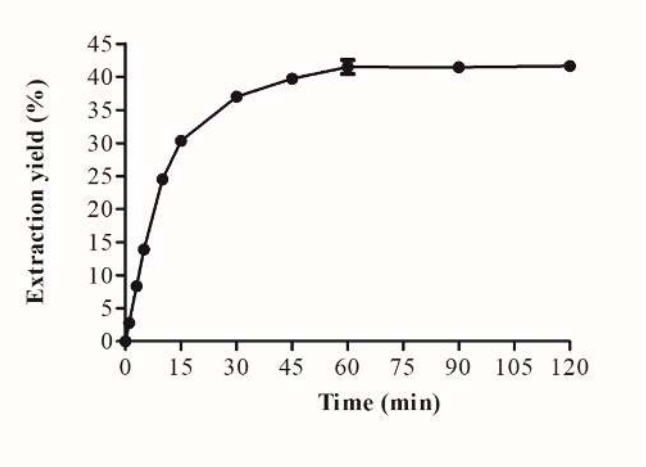

Figure 2. Global curve of extraction of total compounds from cambuí fruit over 120 minutes at a temperature of 50º C, flow rate of 1 mL per min, constant pressure of 200 bar reusing water as extractor solvent.

According to previously reported studies, the dominant mass transfer occurs in the CER period due to the external surface of the particles (vegetable sample) being covered by solutes (bioactive compounds). Therefore, they are easily extractable to the liquid phase (solvent).

The FER period consists of the onset of convection and diffusion mechanisms operating together due to partial exhaustion of the solute from the surface of the vegetative matrix. The REL period is characterized by the complete exhaustion of the solute on the external solid surface, causing resistance to the transfer of mass from the solid phase to the liquid phase [Hall et al., 2018, De Melo et al., 2016, da Silva et al., 2016].

As shown in Figure 2, the CER period (0-29 min) showed the highest extraction capacity. However, the best extraction time was chosen at the end of the FER period, which occurred in about 60 min. The GEC slope was initiated in the FER period, mainly for 30 to 60 min.

After the FER period, we can observe the RLE period (61 to 120 min), characterized by a stabilization of the extraction capacity of the cambuí compounds, as well as the best moment to obtain an optimized extraction time and solvent volume. Thus, according to Figure 2, the best extraction time was 60 min with a solvent volume of 60 mL for each 5 g of cambuí.

Using the inheritance process with water under high pressure showed a good global yield of extraction yield of cambuí biomolecules (Figure 3). As can also be seen in Figure 3, the GYE corresponded to (41.50 ± 1.1%) as well, as the amount of total phenolic compounds were [(92.9 ± 13.1) (mg/g of sample dry)], and total flavonoids were [(10.70 ± 1.4) (mg/g of dry sample)].

Thus, the results found in the present study suggest that water at 200 bar with a temperature of 50 ºC and a flow rate of 1 mL/min allows an efficient obtainment of bioactive molecules from cambuí.

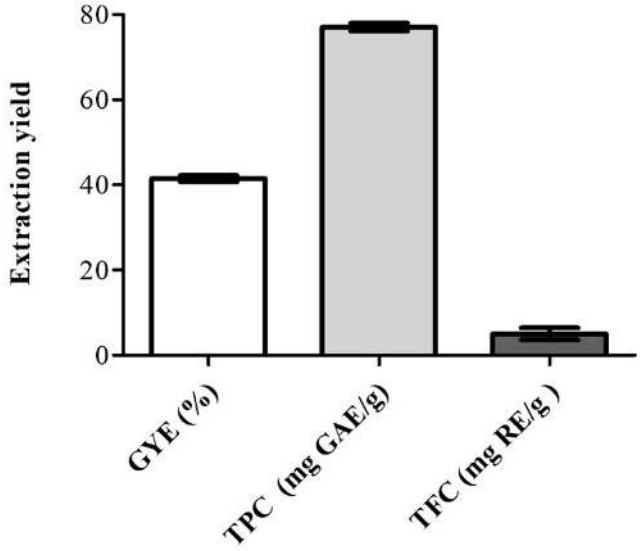

Figure 3. Global yield of extraction (GYE), total phenolic compounds (TPC), and total flavonoid contents (TFC) obtained for 60 min of extraction.

Through the image, it is observed that the values are reported as a mean and standard deviation, in percentage for GYE, milligrams of gallic acid equivalents per gram (mg. GAE/g) of cambuí dry extract for TPC, and milligrams of rutin equivalents per gram (mg.RE/g) of cambuí dry extract for TFC.

Water under high pressure makes it possible to remove considerable amounts of flavonoids and other phenolic compounds, as seen in Figure 3. The values of phenolic compounds found in the present study were higher than the values (mg/100 g of sample) found by Rufino et al., 2010 in aqueous pulp extracts of *Myrtaceae* families, such as Myrciaria cauliflora (3584 ± 90.9), Syzygium cumini (1117 ± 67.1), Eugenia pyriformis (1930 ± 129) and Blepharocalyx salicifolius (2055 ± 75.7).

3.2 Evaluation of antibacterial activity

Plant polyphenols are structurally diverse compounds used for centuries for medicinal purposes, including treating infections. In botanical mixtures, several polyphenols, phenolic acids, flavonoids, tannins, stilbenes, and combinations exhibited significant antibacterial activity against resistant and non-resistant Gram-positive bacteria [Álvarez Martínez et al., 2018].

This information is in line with what was found in the present study ([**?**], in which the aqueous extract of cambuí could inhibit the growth of Enterococcus faecium bacteria.

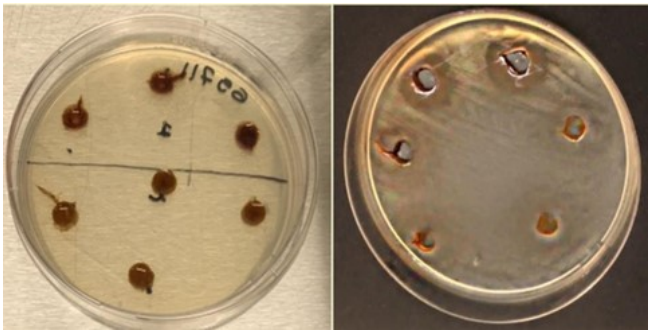

Figure 4. It presents the results of the AST of the EAC against the bacterium Enterococcus faecium and its respective halo of inhibition. Source: The Authors (2021).

The results in Figure 4 show an average inhibition halo for Enterococcus faecium bacteria of (18.4 ± 1.1 mm). Thus, we suggest that the aqueous extract of cambuí is an efficient antibacterial agent to inhibit the growth of nosocomial microorganisms, as it can be a possibility for the treatment against this pathogen that can cause a variety of infections, including endocarditis, urinary tract infection, prostatitis, intra-abdominal infection in addition to concurrent bacteremia [Mališová et al., 2021], the use of the aqueous extract of cambuí is intended to be an alternative treatment compared to existing conventional antibiotics.

Table 1 shows the sensitivity and resistance profile of Enterococcus faecium bacteria against cambuí extract (EAC) and some commercial antibacterial agents.

The interpretation of drug susceptibilities allowed inferring the susceptibility and resistance profile of Enterococcus faecium isolated against different classes of tested antimicrobials and the presence of multidrug resistance mechanisms. Table 1 shows the susceptibility profile of Enterococcus faecium bacteria against other antibacterial agents.

As shown in Table 1, of the 10 commercial antimicrobial agents tested, 7 efficiently inhibited the growth of Enterococcus faecium bacteria. However, it was also resistant to Ampicillin, Imipenem, Vancomycin, and Teicoplanin. According to Table 1, the bacterium Enterococcus faecium presents multidrug resistance mechanisms.

However, as shown in Figure 4 and Table 1, EAC containing flavonoids and other phenolic compounds inhibited the growth of Enterococcus faecium bacteria. Thus, it is worth mentioning that the extract was obtained from an edible fruit,

Table 1. Susceptibility profile of *Enterococcus faecium* bacteria against different antibacterial agents.

Antimicrobial agents	Enterococcus faecium
EAC	Sensitive
Ampicillin	Resistant
Gentamicin	Sensitive
Streptogramins	Sensitive
Imipenem	Resistant
Vancomycin	Resistant
Teicoplanin	Resistant
Linezolid	Sensitive
Daptomycin	Sensitive
Tetracycline	Sensitive
Sinercid	Sensitive

Source: The authors (2021).

which, in adequate concentrations, can act as a functional food or food additive for juices and other types of beverages. Based on our results from the present study, we can suggest that the main antibacterial molecules of cambuí and its obtained extracts are related to its phenolic compounds.

According to the literature, polyphenols have a mechanism of action that is associated with their chemical structure, which can cause morphological changes in bacteria, such as deterioration of the bacterial cell wall, influence protein biosynthesis, alter metabolic processes in bacterial cells, and inhibit ATP and DNA synthesis [Efenberger-Szmechtyk et al., 2021].

Plant polyphenols are promising sources of antibacterial agents, alone or in combination with existing antibiotics, for the development of new antibiotic therapies [Álvarez Martínez et al., 2018] .

4. Conclusion

In the present study, we concluded that the best extraction time for bioactive molecules from cambuí was 60 min using 60 mL of water at a temperature of 50 °C, pressure of 200 bar, and flow rate of 1 mL per min-1, for each 5 g of dry sample.

The aqueous extracts of cambuí showed a relevant overall extraction yield (41.50 ± 1.1%), as well as being rich in total flavonoids (10.70±1.40 mg/g of dry sample) and other total phenolic compounds (92.9 ± 13.1 mg/g dry sample).

The cambuí extracts obtained by pressing hot water are a viable alternative for thwarting the growth of gram-positive bacteria, such as Enterococcus faecium, which is resistant to Ampicillin, Imipenem, Vancomycin, and Teicoplanin. Extracts obtained can be added to functional beverages, such as juices and teas, to enhance their nutritional value.

However, other studies must be conducted to verify the minimum inhibitory concentration and analyze a possible broad spectrum of antimicrobial action, mechanisms of action presented by the extract obtained, and pharmacodynamic and pharmacokinetic studies in vitro and in vivo. In general, cambuí is a food that contains antibacterial molecules and can be a relevant alternative against bacterial resistance.

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